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14. ABSTRACT

We have shown using knockout mice and other methods that the neuronal/synaptic receptor protein known as EphB1 is a central player in nerve injury-induced chronic neuropathic pain as well as the related pain symptoms associated with the withdrawal from opioid/morphine addiction. Our hypothesis is that EphB1 participates in pain caused by nerve damage and opioid withdrawal through the ability of its extracellular domain to form protein-protein interactions with the NR1 subunit of the NMDA receptor and inappropriately strengthen the synapses and neural circuits in the spinal cord that transmit pain signals up into the brain. Our project is to carry out high-throughput screens (HTS) to identify small molecular weight drug-like compounds that antagonize the EphB1:NR1 protein-protein interaction. In year 1 of the project we have cloned, expressed, and purified the unique protein tools needed for the project. We have also worked to develop protein-protein interaction assays necessary for the HTS.

15. SUBJECT TERMS

Chronic neuropathic pain, opioid addiction, synaptic plasticity, EphB1 receptor, NMDA receptor, drug discovery

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1. **INTRODUCTION:**

As described in the submitted application, our plan is to carry out high throughput screens of large libraries of small drug-like compounds to identify those that diminish or eliminate the EphB1-NR1 protein-protein interaction. Damage to pain sensing peripheral nerves following traumatic injury or other insult, such as diabetic neuropathy or bone cancer growth, strongly elevates the protein-protein interaction between EphB1 and the NR1 subunit of the NMDA receptor in postsynaptic structures of spinal dorsal horn neurons and this drives an increase in long-term potentiation (LTP) of these synapses. The increased LTP triggers enhanced transmission of pain impulses that project into the brain, leading to classic neuropathic pain states. Similar mechanisms likely explain the severe withdrawal symptoms from the highly addictive opioid family of drugs (e.g. morphine, hydrocodone, and heroin). Whether due to nerve damage and/or withdrawal from opioid-based drugs, neuropathic pain is a serious problem faced by active military personnel, veterans of past service, as well as the general civilian population. Our project is to conduct biochemical and cell-based experiments using purified EphB1 and NR1 ectodomain protein domains to screen and identify small molecular weight drug like chemicals that disrupt the EphB1-NR1 protein-protein interaction. The plan is to screen two different types of drug libraries, one a highly diverse chemical based library and the other a complex peptoid/peptide based library. If successful, we anticipate discovering new smart drugs that will be much more effective at preventing or reversing pain and we hope to begin testing such compounds in an animal model of chronic pain.

2. **KEYWORDS:**

Nerve injury pain, chronic pain, neuropathic pain, opioid addiction, EphB1 receptor, NMDA receptor NR1 subunit, LTP, protein-protein interaction, antagonist, high throughput screen, drug discovery

3. **ACCOMPLISHMENTS:**
   - **What are the major goals of the project?**

Aim 1: To screen a library of small drug-like compounds to identify small molecular weight chemicals that antagonize the EphB1-NR1 protein-protein interaction.

Aim 2: To screen peptoid libraries for short peptide-like compounds that bind to the extracellular domain of EphB1 and antagonize the EphB1-NR1 protein-protein interaction.

Aim 3: To carry out an initial pilot test of the strongest antagonistic compound identified for its ability to reduce nerve injury-induced neuropathic pain and opioid withdrawal-induced pain enhancement in established rodent animal models.

   - **What was accomplished under these goals?**

In year 1 we focused on developing the unique reagents needed to develop and conduct the high throughput drug screens outlined in Aims 1 and 2. It was important for the project to get off to a good start by generating new plasmid vectors that would allow us to express and purify in bacteria large amounts of each of the two EphB FN3 repeats (each ~90 amino acids long) as isolated proteins fused to GST (GST-EphB1-FN3a, GST-

EphB1-FN3b, and GST-EphB1-FN3a+b) as well as the NR1 extracellular N-terminal domain (NTD) protein fused to His tag (NR1-NTD-His). Our original experiments used the entire full EphB1 (~550 amino acids) and NR1 (~500 amino acids) ectodomains, and this resulted in only a weak protein-protein interaction. We thus decided to reduce the size of the proteins and focus on pruifying only the segments needed to mediate the EphB1-NR1 protein-protein interaction, the FN3 domains of EphB1 and NTD domain of NR1, and anticipated this would provide for stronger interactions as we develop the drug screens. We also generated at the same time plasmid vectors to express the isolated FN3a and FN3b domains of the highly related EphB2 receptor (GST-EphB2-FN3a, GST-EphB2-FN3b, and GST-EphB2-FN3a+b) as this protein also forms protein-protein interaction with NR1 and may provide better binding results. As a major accomplishment in year 1, we successfully cloned all the new plasmid expression vectors and have expressed and purified in bacteria large milligram quantities of all GST-tagged EphB1 and EphB2 FN3 domains as well as multiple His-tagged versions of the NR1 NTD necessary for experiments/assays. This work described above was carried out by technician Ryan Gleber under the guidance of Dr. Henkemeyer.

In year 1, we also began developing protein-protein interaction assays using the large-scale purified GST-EphB-FN3 and NR1-NTD-His proteins. Development of protein-protein interaction assays are crucial for accomplishing the goals of the proposal. Our initial experiments focused on biochemical pulldown and ELISA assays using the GST fusion proteins as baits and NR1-NTD as prey. As discussed in Y1Q3 Progress Report, our ability to detect with in vitro assays the protein-protein interaction between the isolated EphB receptor FN3 domains and the NR1 ectodomain needed for AlphaScreen assay development is proving to be a challenge (see below). These types of protein-protein interaction experiments can be very technically demanding as we need to demonstrate robust protein associations between the FN3 domains and the NR1 protein. There are a number of potential reasons why the interactions are difficult to detect and we have been addressing these possibilities in the past quarter (see below). Nevertheless, even though we have encountered technicial issues with detecting robust protein-protein interactions, our most recent data indicates we will be able to work through these problems and will soon begin setting up the actual drug library screening assays. This work described above was carried out by technician Melody Karsi under the guidance of Dr. Henkemeyer.

In year 1 we have also worked on refining the cell-based assays we will use to detect and measure the EphB1-NR1 protein-protein interaction using full length transmembrane form of the EphB1 receptor and NR1 protein co-transfected in mammalian cells growing in culture. These ongoing experiments will be very important in our analysis and validation of the antagonistic activity of drug compounds that will be identified from the drug library screening. This work on cell-based assays is being carried out by a new postdoctoral fellow who recently joined the laboratory, Dr. Asim Bepari, under the guidance of Dr. Henkemeyer.

- **What opportunities for training and professional development has the project provided?**

Dr. Henkemeyer provides day-to-day, one-on-one guidance and training of all individuals working on the project, including Ryan Gleber, Melody Karsi, lab manager Frances Sprouse, and Dr. Asim Bepari. Of note, Ryan Gleber, recently left the Henkemeyer

laboratory and moved to California to begin medical school at UC-San Diego. UT Southwestern also provides a large and diverse faculty and seminars for advanced professional development.

- **How were the results disseminated to communities of interest?**

As we are just in the beginning stages of the project, we have not publically disseminated any of our data.

- **What do you plan to do during the next reporting period to accomplish the goals?**

During the next reporting period we hope to advance our ability to detect protein-protein interactions between the purified EphB1 and NR1 proteins and start screening the chemical libraries. We also will better develop cell-based assays to vigorously detect the protein-protein interaction between full-length EphB1 and NR1 proteins in order to help validate primary hits from the library screens.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**

Our protein-protein interaction tests using bacterial purified proteins show that we can detect the protein-protein association between purified EphB FN3 domains and the NR1-NTD. In these initial experiments we may have also revealed some novel and interesting features of the interactions that suggest they may be quite complex and have unique EphB subclass specificity. While adding to our challenge at demonstrating robust EphB-NR1 interactions, I believe these results are giving us unique scientific insight into the extraordinary complexities of these special proteins and the interactions they are capable of as they control synaptic plasticity.

- **What was the impact on other disciplines?**

While our studies are still in their early stages, I believe our work will impact general fields of receptor biology and cell-cell signaling. Very little is known about the various protein interacting partners of FN3 domains, even though this domain is found in a wide range of membrane spanning molecules, 170 different proteins in humans, and is often repeated many times in any particular molecules (i.e. there are two FN3 domains in Eph receptors, which we term FN3a and FN3b). Our studies on the Eph FN3 domains ability to bind NR1-NTD may form a model system that others may utilize in their studies of other FN3 domain-containing proteins.

What was the impact on technology transfer?

Again, while our studies are still in their early stages, we certainly hope we are able to identify strong antagonists of the EphB1-NR1 protein-protein interaction. If we are indeed successful, there will be great potential to impact technology transfer as we would have our hands on a new class of drug-like compounds that could be further developed into actual new drugs to treat and/or prevent chronic pain conditions caused by nerve injury and/or withdrawal from opioid abuse.

- **What was the impact on society beyond science and technology?**

As stated above, our high-risk research has the potential to product a high-impact result, new drugs to combat pain and addiction.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**

No major changes to report.

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

While we have purified large amounts of the various proteins, the protein-protein interaction experiments between the isolated EphB receptor FN3 domains and the NR1 ectodomain needed for AlphaScreen assay development are proving to be a challenge. These types of protein-protein interaction experiments can be very technically demanding as we need to demonstrate robust protein associations between the FN3 domains and the NR1 protein. There are a number of potential reasons why the interactions are difficult to detect and we have been addressing two key possibilities in the past quarter.

One possibility is that a ternary complex is needed for robust interaction that involves: (1) the EphB FN3 domain, (2) the NR1 ectodomain, and (3) the EphB ligand known as ephrinB2 (EB2). Here, we have added soluble EB2 ectodomains to the protein-protein interaction assays to determine if that is what is needed to obtain robust association between EphB FN3 domain and NR1 ectodomain. The results indicate that the ability of NR1 to interact with EphB1-FN3b (and EphB2-FN3a) depends on the presence of EB2. Interestingly, our experiments indicate the ability of EphB1-FN3a to interact with NR1 may actually be inhibited by addition of EB2. This suggest complex biochemical interactions are at play, adding to our scientific curiosity and giving us things to think about as we dig deeper into the protein interactions that drive synaptic activity and plasticity in the brain and spinal cord.

Another possibility is information from a colleague that indicates the EphB FN3 domain may become phosphorylated on tyrosine residues and they have suggested this may aid the interaction with NR1 ectodomain. We therefore phosphorylated our purified FN3 domain protein in vitro using commercially available protein kinases (EGF receptor and FGF receptor kinase domains) to see if this will enhance the FN3-NR1 associations. In both cases, adding kinase to phosphorylate the FN3 domain did not appear to increase the FN3-NR1-NTD protein-protein interaction. In fact, phosphorylation if anything appears to actually decrease the protein-protein interactions.

In summary, I believe we are working through these challenges and getting better at detecting stronger interactions between the FN3 and NR1 domains. It just seems that relatively minor tweeks and refinements to the interaction protocols and washing steps, combined with skills gained by repeating the experiments over multiple times has helped us achieve consistency. Thus, while proving to be technicially challenging and causing some delays in our ability to push forward on the actual AlphaScreen and peptoid screens, we are gaining experience on working with FN3 and NR1 proteins and will overcome these difficulties. We are now confident we can begin setting up the high throughput assays necessary for the screening of drug compound libraries.

- **Changes that had a significant impact on expenditures**

None, resources have been conserved as we work our way on refining our protein-protein interactions.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

N/A

- **Significant changes in use or care of human subjects**

N/A

- **Significant changes in use or care of vertebrate animals.**

N/A

- **Significant changes in use of biohazards and/or select agents**

N/A

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**

Nothing to report.

   - **Website(s) or other Internet site(s)**

Nothing to report.

   - **Technologies or techniques**

See above.

   - **Inventions, patent applications, and/or licenses**

Nothing to report.

   - **Other Products**

Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**
   - **What individuals have worked on the project?**

Name:	Mark Henkemeyer
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Worked on all aspects of project.
Funding Support:	
Name:	Frances Sprouse
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Lab manager, assisted on all aspects of project.
Funding Support:	
Name:	Ryan Gleber
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Cloned plasmids, expressed and purified proteins from bacteria.
Funding Support:	
Name:	Melody Karsi
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Conducted biochemical protein-protein interactions assays experiments on purified proteins.
Funding Support:	
Name:	Asim Bepari
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Conducted cell-based experiments on EphB1-NR1 protein-protein interaction using mammalian cells.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS:**

COLLABORATIVE AWARDS:

N/A

- **QUAD CHART:**

See attached

9. **APPENDICES:**

N/A

EphB1 as a Novel Drug Target to Combat Pain and Addiction

1115013

Clinical & Rehabilitative Medicine

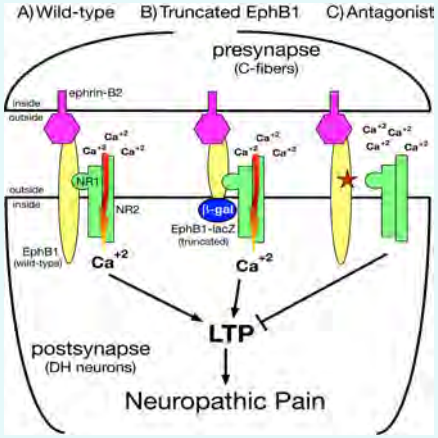
PI: Dr. Mark Henkemeyer **Org:** University of Texas, Southwestern Medical Center **Award Amount:** $1,385,682

Study/Product Aim(s)

- Aim 1: To screen a library of small drug-like compounds to identify those that antagonize the EphB1-NR1 protein-protein interaction.
- Aim 2: To screen on-bead peptoid libraries for compounds that bind to the extracellular domain of EphB1 and antagonize the EphB1-NR1 protein-protein interaction.
- Aim 3: To carry out an initial pilot test of the strongest antagonistic compound identified for its ability to reduce nerve injury-induced neuropathic pain and opioid withdrawal-induced pain enhancement in established rodent animal models.

Approach

AlphaScreen test tube based (Aim 1) and on-bead peptoid cell-based (Aim 2) screens, will be followed by biochemical tests to identify and validate EphB1-NR1 antagonist compounds.



EphB1-NR1 interaction is important for pain. Wild-type (A) and intracellular truncated mutant (B) mice exhibit nerve injury-induced neuropathic pain due to the ability of EphB1 to form a protein-protein interaction with the NR1 subunit of the NMDA receptor ion channel complex and induce LTP in dorsal horn neurons. In (C), we predict that small chemicals that antagonize the EphB1-NR1 protein-protein interaction (red star) can be found and will block LTP formation and neuropathic pain.

Accomplishments: We have cloned expression vectors, purified EphB1 and NR1 ectodomain proteins, and continue to work on developing the protein-protein interactions assays needed for HTS screens and validation tests.

Timeline and Cost

Activities CY	14	15	16	17
Develop Alpha/Peptoid screens				
Complete Alpha/Peptoid screens				
Validate antagonistic compounds				
Animal tests of best compound for ability to minimize pain				
Estimated Budget ($K)	**$435K**	**$434K**	**$517K**	**$000**

Updated: (UTSW, September, 17, 2015)

Goals/Milestones

CY14 Goals – Develop protein reagents and interaction assays

☐Clone vectors to make EphB1/FN3 and NR1/NTD domains

☐Purify large amounts of EphB1 and NR1 proteins from bacteria

☐Establish EphB1-NR1 AlphaScreen assay and other assays

CY15 Goals – Conduct HTS screens and begin validation

☐Optimize protein-protein HTS interaction assays

☐Begin HTS screens

☐Initiate biochemical and cell-based tests of promising lead hits

CY16 Goals – Complete validation and conduct pilot tests in rodents

☐Continue biochemical and cell-based tests of promising lead hits

☐Test the most promising lead compound in animal models of pain

☐**Comments/Challenges/Issues/Concerns**

- Work with these proteins has been difficult, we have conserved funds as we overcome these challenges and have adjusted the timeline.

Budget Expenditure to Date

Projected Expenditure: $108,718 (Y1Q4) / $434,872 (Y1Q1-Y1Q4)

Actual Expenditure: $112,219 (Y1Q4) / $372,063 (Y1Q1-Y1Q4)